



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>5</sup> :  G01N 33/53</p>	<p>A1</p>	<p>(11) International Publication Number: <b>WO 90/04786</b>  (43) International Publication Date: 3 May 1990 (03.05.90)</p>
<p>(21) International Application Number: PCT/US89/04320 (22) International Filing Date: 10 October 1989 (10.10.89) (30) Priority data: 258,894 17 October 1988 (17.10.88) US (60) Parent Application or Grant (63) Related by Continuation US 258,894 (CIP) Filed on 17 October 1988 (17.10.88) (71) Applicant (for all designated States except US): MOLECULAR DEVICES CORPORATION [US/US]; Menlo Oaks Corporate Center, 4700 Bohannon Drive, Menlo Park, CA 94025 (US).</p>		<p>(72) Inventors; and (75) Inventors/Applicants (for US only): OLSON, John, D. [US/US]; 1055 Lois Avenue, Sunnyvale, CA 94087 (US). ZUK, Robert, F. [US/US]; 1536 Los Montes, Burlingame, CA 94010 (US). ARMENTA, Richard, D. [US/US]; 1085 Ticonderoga Drive, Sunnyvale, CA 94087 (US). BURKE, Charles, R. [US/US]; 945 Old Trace Road, Palo Alto, CA 94306 (US). KUNG, Viola, T. [US/US]; 1055 Lemon Street, Menlo Park, CA 94025 (US). SHELDON, Edward, L., III [US/US]; 788 17th Avenue, Menlo Park, CA 94025 (US). (74) Agents: SARUSSI, Steven, J. et al.; Allegretti &amp; Witcoff, Ltd., 10 South Wacker Drive, Chicago, IL 60606 (US). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  Published With international search report.</p>
<p>(54) Title: HAPTEN DERIVATIZED CAPTURE MEMBRANE AND DIAGNOSTIC ASSAYS USING SUCH MEMBRANE</p> <p>(57) Abstract</p> <p>The present invention encompasses a capture membrane comprising a porous filter membrane having a hapten bound directly or indirectly to the membrane wherein complexes formed by specific binding having an anti-hapten bound to a binding member of the specifically binding complex are removed from a solution by the hapten as the solution passes through the membrane. In the preferred embodiment biotin is the hapten and avidin or streptavidin is the anti-hapten.</p>		

BEST AVAILABLE COPY

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LJ	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

HAPTEN DERIVATIZED CAPTURE MEMBRANE AND  
DIAGNOSTIC ASSAYS USING SUCH MEMBRANE

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of pending U.S.  
5 Patent Application Serial No. 258,894, filed on October 17, 1988.

Field of the Invention

This invention relates to a diagnostic reagents, capture  
membranes, which are useful in removing specifically binding  
complexes from solutions and assay methods using such reagents. The  
10 specifically binding complexes include antigen/antibody complexes,  
DNA, anti-DNA (antibody to DNA) or Single Strand DNA Binding Protein  
(such as SSB from *E. coli*), DNA, DNA hybrids, RNA, RNA hybrids, and  
similar specifically binding complexes.

The Related Art

15 There are extensive teachings in the art of diagnostic assays  
and reagents involving specifically binding complexes.  
Antigen/antibody reactions are widely used to determine antigen and  
antibodies. The labeling of members of these complexes with  
detectable markers such as enzymes or florescent dyes is well known.  
20 The binding of antigen or antibodies and solid supports as a means of  
removing complexes from solutions is also known. The use of haptens  
such as biotin and anti-haptens such as streptavidin in diagnostic  
assay is extensively discussed in a review article that appears in  
Analytical Biochemistry 171, 1-32 (1988).

25 Biotin attached to a solid-support is described in U.S. Patents  
4,282,287, 4,478,914, and 4,656,202. These patents describe precise  
layering technique wherein biotin is first attached to a solid-  
surface and the subsequent application of successive layers of avidin  
and extender results in a controlled modification of surface  
30 characteristics.

European Patent No. 87,307,850.5 is directed toward a method for  
routine plant-virus diagnosis which includes biotin attached to a  
macro-molecule that is conjugated to a sample of probe DNA. The  
probe containing compound is applied to a solid-matrix which has a  
35 test sample of DNA derived from plant tissue immobilized thereon.  
The presence of the target sequence is determined by washing the

-2-

matrix with enzyme linked avidin followed by assaying for enzyme activity associated with the matrix.

U.S. Patent No. 4,467,031 describes an enzyme-immunoassay which utilizes the biotin-avidin system as a convenient and stable linking  
5 group for connecting a reporter enzyme to an antibody.

U.S. Patent No. 4,228,237 describes the use of the biotin-avidin system in a method for detection and determination of ligands. A surface having an antibody for the ligand of interest attached thereto is reacted with a sample of the ligand followed by a second  
10 ligand specific antibody that is conjugated with biotin. This complex is then reacted with an avidin conjugated enzyme and the results are determined by measurement of enzyme activity.

U.S. Patent No. 4,656,025 describes a screening assay for tumor globulin. A tumor globulin-biotin conjugate on ELISA plates is  
15 reacted with avidin conjugated enzyme and quantification of the tumor globulin bound to the plate is determined by the application of the appropriate chromogenic substrate thereto.

U.S. Patent No. 4,535,057 describes an immunoassay having biotin conjugated to a solid support through an antibody-virus complex.  
20 This biotin-antibody virus complex is then reacted with avidin conjugated to a reporter group or a label and the presence of the label associated with the surface is indicative of the presence of virus in the sample.

U.S. Patents 4,727,019, and 4,632,901 describe an immunoassay  
25 wherein avidin is attached to a solid support and binds a ligand present in the sample to the support. U.S. Patent No. 4,298,685 describes a diagnostic reagent that also involves avidin immobilized on a solid support. U.S. Patent No. 4,582,810 describes a detection system wherein a suspension of particles having avidin covalently  
30 bound thereto reacts with a biotin-antibody complex to form a complex which results in a flocculent appearing solution.

U.S. Patent No. 4,550,075 describes a method for ligand determination based on the biotin-avidin system without any solid support.

35 U.S. Patent No. 4,486,530 describes an immunometric assay process that comprises a ternary complex of an antigenic substance,

**SUBSTITUTE SHEET**

-3-

and a first and second antibody bound to the antigen in which the complex is removed from solution by filtering through a membrane.

Clinical Chemistry, 34, No. 8, p. 1585 (1988) describes a monoclonal antibody based noncompetitive avidin-biotin assay for  
5 luteinizing hormone (LH) in urine.

U.S. Patent No. 4,778,751, describes a method for measuring antigens which comprises: forming in a liquid phase reaction a soluble complex wherein an antigen (Ag<sub>1</sub>), antibody (Ag<sub>1</sub>) or hapten (H) is linked through, respectively, a specific antibody (Ab),  
10 antigen (Ag) or anti-hapten (Anti-H), to a matrix which is soluble in the liquid phase and carries a ligand (X), the matrix capable of being chemically attached to more than one specific antibody (Ab), antigen (Ag) or anti-hapten (Anti-H); forming an insolubilized complex comprising a solid support linked to the ligand (X) of the  
15 soluble complex through an anti-ligand (Y), the insolubilized complex carrying a label (Z) linked to the antigen (Ag<sub>1</sub>) through an anti-antigen (Anti-Ag<sub>1</sub>), to the antibody (Ab<sub>1</sub>) through an anti-antibody (Anti-Ab<sub>1</sub>) or to the hapten (H); washing the insolubilized complex;  
20 and observing the washed insolubilized complex for the presence of the label (Z) wherein the presence of the label (Z) is an indication of the level of the antigen (Ag<sub>1</sub>), antibody (Ab<sub>1</sub>) or hapten (H) in the sample.

European Patent Application No. 86111379.3 describes multilayer immunoassay test devices involving the use of labeled reagents  
25 comprising a chemical group having a detectable physical property such as fluorescence or color.

European Patent Application No. 88.308164.8 describes a method for determination of single-stranded DNA based on the binding of a single-stranded DNA to a single-stranded DNA-binding protein to which  
30 is bound a solid support.

Molecular Immunology, 34: 221-230 (1989) describes an ELISA system involving immobilization of biotinylated CAbS through linkage by streptavidin to biotinylated carrier proteins absorbed on polystyrene. The present invention provides technology for removal  
35 of specifically binding complexes from a solution and differs from the prior art in that the reagent of this invention is a porous

-4-

membrane with a hapten preferably biotin bound directly or indirectly to the membrane.

-5-

SUMMARY OF THE INVENTION

The present invention encompasses a capture membrane comprising a porous filter membrane having a hapten preferably biotin bound directly or indirectly to the membrane wherein specifically binding  
5 complexes having an anti-hapten preferably avidin or streptavidin bound to a binding member of the specifically binding complex are removed from a solution by the hapten when the solution flows through the membrane.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention comprises a porous membrane or filter to which is bound a hapten; this membrane or filter being capable of filtering a solution that may contain a specifically binding complex having an anti-hapten bound to a binding member of the specifically binding complex.

The material of the membrane or filter is selected from material to which protein or other macromolecule can be adhered. A variety of materials may be used. Those skilled in the art will appreciate that porous membranes made of nylon, cellulose acetate, polyolefin, polyacrylamide, nitrocellulose or other porous materials may be employed in the present invention. Other synthetic or naturally occurring materials which will adhere a protein or other macromolecule may also be used. A preferred membrane is made from nitrocellulose.

Haptens are substances which do not elicit antibody formation unless complexed to macromolecules and which may be employed in the present invention as specific organic materials for which specific binding substances can be provided. Antibodies to haptens can be formed by binding the hapten to a protein to elicit an antibody response. A specific binding substance is any substance or group of substances having a specific binding affinity for the hapten to the exclusion of other substances. The employed hapten must be able to bind to a protein or other macro-molecule directly or through an extended linking group. Examples of haptens which may be used according to the instant invention include steroids such as estrone, estradiol, testosterone, pregnanediol and progesterone; vitamins such as B<sub>12</sub>, biotin and folic acid; triiodothyronine, thyroxine, histamine, serotonin, digoxin, prostaglandins, adrenalin, noradrenalin, morphine, vegetable hormones and antibiotics such as penicillin.

When the hapten is a substance having a naturally occurring receptor, the receptor can be utilized as the anti-hapten provided the receptor can be isolated in a form specific for the hapten. Illustrative haptens which have naturally occurring receptors include thyroxine, many steroids, polypeptides, such as insulin, angiotensin,



-7-

biotin and many others. Receptors for this class of haptens are usually proteins or nucleic acids.

Extended linking groups are groups that will bind the hapten to the protein or macro-molecule in such a way that the hapten has better access to the anti-hapten. Extended linking groups useful in the present invention include succinylated polylysine, dextran, polyethylene glycol, and preferentially a polyamido ether extending group. These extended linking groups may be used separately or in combination to obtain extended linking groups of varying lengths and binding properties. Extended linking groups are preferred for use with serum samples especially lipemic serum samples. Evidently, there are interfering substances in serum samples, the interference from which is overcome by the extended linking group. Where an extended linking group is not needed, a hapten such as biotin, without an extended binding group is bound to a functional group on a membrane or to a function group on a protein which can be disbursed on the membrane.

The extended linking group must be able to bind to the protein or macromolecule. Preferentially the extended linking group having an hapten bound to one end will be bound to the protein or macro-molecule with an amide bond; the amine of the amide bond arising from the protein and the carboxyl of the amide bond arising from the carboxy terminus of the extender group. Free carboxyl or hydroxyl groups on proteins can likewise be used.

The proteins and macro-molecule of the present invention include, but are not limited to, bovine serum albumin (BSA), bovine gamma globulin, and fibrinogen.

The complex produced by specific binding which is removed from the solution as it passes through the filter comprises two binding members and an antigen, with the proviso that one binding member is bound to an anti-hapten and the other binding member is bound to a labelling group.

The anti-haptens bound to the binding members of the present invention comprise the molecules described above that act as receptors to the above mentioned haptens. Antibodies are preferred binding members which are conveniently labeled with enzymes or

-8-

fluorescent dyes and are also conveniently bound to anti-haptens such as avidin or streptavidin or antibodies to haptens.

This embodiment of the invention can be viewed as follows:

Capture Membrane

Complex

5 membrane - hapten . anti hapten - Ab Ag Ab-(labeled)  
 membrane - (biotin) . (streptavidin)-Ab' Ag' Ab(enzyme)

Persons having skill in the art will recognize that capture membranes may also be employed in sequential assays. In such an assay, a series of filtration steps is used to capture and detect a  
 10 substance to be determined. Such an assay may have many advantages over standard, non-sequential assays.

For example, a solution of anti-hapten could be filtered through a haptenated porous filter membrane, whereby the anti-hapten would be captured on the membrane. A solution containing an anti-hapten  
 15 binding substance would subsequently be filtered through the membrane, thereby capturing the anti-hapten binding substance. This anti-hapten binding substance may be a hapten or other substance which binds to an anti-hapten. The anti-hapten binding substance preferably is modified with binding sites for a substance to be  
 20 determined. Filtering a solution of a substance to be determined through the membrane will capture the substance to be determined on the membrane. The substance to be determined may then be detected on the membrane by filtering and labeling the substance to be determined with a detectable label and detecting the label on the membrane. The  
 25 label may then be detected by various methods in the art. For example, an electrode which is a semiconductor may be employed in one embodiment of the present invention.

Persons having skill in the art will recognize that many combinations of sequential assays may be performed. Complexes may be  
 30 formed between anti-haptens, haptens, antigens, antibodies, substances to be determined and detectable labels. These complexes may have one or more components. Thus, assays may be designed to avoid interference from specific substances.

This embodiment of the invention can be viewed as follows:

-9-

Capture MembraneComplexmembrane-hapten-  
antihapten

. hapten-Ab Ag Ab-(labeled)

5 membrane-(biotin)  
(Streptavidin)

. biotin-Ab' Ag' Ab-(enzyme)

Antibodies may be determined in a manner similar to that for an antigen. Complexes may be formed between an anti-hapten, an antibody, an antigen bound to a hapten or anti-hapten and another antigen bound to a labeling group.

- 10 The haptens and anti-haptens comprise those molecules described above as haptens and anti-haptens. Antigens may be conveniently labeled with enzymes or fluorescent dyes and are also conveniently bound to haptens or anti-haptens.

These embodiments of the invention may be viewed as follows:

15 Capture MembraneComplex

membrane-hapten

. anti-hapten hapten-Ag Ab Ag-labeled

membrane-(biotin)

. (streptavidin)(biotin)-Ag Ab Ag-(enzyme)

- 20 The antibodies employed in the present invention may be either polyclonal or monoclonal antibodies and are produced in response to the target antigen of the assay. Methods for the production of antibodies to various biological substances are well known in the art.

- 25 The antigens targeted by the assay include, but are not limited to antigens such as IgE, prostatic acid phosphatase, prostate specific antigen, alphafetoprotein, carcinoembryonic antigen, luteinizing hormone, creatine kinase MB, Human Chorionic Gonadotropin (HCG) and other antigens in serum, plasma, urine, or other liquid media.

- 30 Polydeoxyribonucleotides can be determined by reactions with single strand DNA binding protein (SSB) and anti-DNA antibodies. Thus, various combinations of labeled SSB or anti-DNA and biotinylated SSB or anti-DNA are employed. In this embodiment streptavidin is bound to the biotinylated SSB or anti-DNA so that the complex can be bound to the capture membrane having biotin. In place  
35 of SSB, an oligonucleotide probe may be used to detect DNA. The

**SUBSTITUTE SHEET**

-10-

article in Biochemistry, 25:21 (1986) describes the large scale over production of single-strand binding protein (SSB) from E. coli. Monoclonal antibodies to DNA have been used to measure DNA in biological fluids, Journal of Immunological Methods, 88, (1986) 185-192.

These embodiments of the invention can be viewed as follows:

<u>Capture Membrane</u>	<u>Complex</u>
membrane-biotin	• streptavidin-biotin-anti-DNA/ DNA/SSB-enzyme
10 membrane-biotin	• streptavidin-biotin-SSB/DNA/ anti-DNA-enzyme
membrane-biotin	• streptavidin biotin-oligonucleotide probe/DNA/ oligonucleotide probe-enzyme

Labeling groups that may be employed in the present invention include enzymes, fluorescent labels and radionuclides. The preferred label is an enzyme that is linked to the antibody at a position which does not interfere with the binding of the antibody to the antigen. Thus, the enzyme should possess potentially reactive groups to which the antibody can be coupled without destroying enzyme activity and should not occur naturally to an appreciable extent in the liquid to be assayed for the specific biological substance. In addition, the enzyme should have a relatively long shelf life, a high specific activity and also be capable of being easily assayed, for example, with a visible light spectrophotometer.

Examples of enzymes which may conveniently be employed in the process of the present invention are, malate dehydrogenase, lipase, delta-5-ketosteroid isomerase, yeast alcohol dehydrogenase, yeast glucose-6-phosphate dehydrogenase, alpha glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, and more preferably, urease. Normally, it is preferred that the enzyme be in a pure form, free of contaminating proteins.

The preparation of the enzyme-labelled biological substances for use in the present invention can be accomplished in various ways known in the art. Examples of the coupling of biological substances

-11-

to enzymes are described in, for example, L. A. Steinberger, Immunocytochemistry, Prentice Hall, New Jersey (1974).

Although a radionuclide such as  $^{125}\text{I}$  or  $^{32}\text{P}$  may also be used as the label non-radioactive labels are preferred.

- 5        After filtering the solution suspected of containing the antigen in a specifically binding complex through the porous filter membrane, the presence of labelled antibody on the porous membrane is then determined as an indication of the presence of the target antigen in the sample. In the case of an enzyme label this may be done by
- 10       addition of a solution of a color forming substrate to the porous member to allow the substrate to react with the enzyme. Determinations can conveniently be made by the devices and methods described in U.S. Patents 4,591,550, 4,704,353, and U.S. Patent Application 876,925, filed June 20, 1986 and assigned to the same
- 15       assignee as this application.

The invention is illustrated further by the following examples which are not to be construed as limiting the invention in scope or spirit to the specific procedures described in them.

---

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the improved efficiency of the biotin membrane (BXL-BSA, biotinylated-Bovine Serum Albumin) versus the avidin (Streptavidin) membrane.

5        FIG. 2 illustrates the efficiency of capture of streptavidin at various densities of biotin using two different types of membrane and two different flow rates.

FIG. 3 illustrates the capture efficiency of 0.8 $\mu$  nitrocellulose filter at various concentrations of BXL-BSA on the membrane and at  
10        various Streptavidin flow rates.

FIG. 4 illustrates the determination of TSH.

FIG. 5 is a standard curve generated by the dual probe assay.

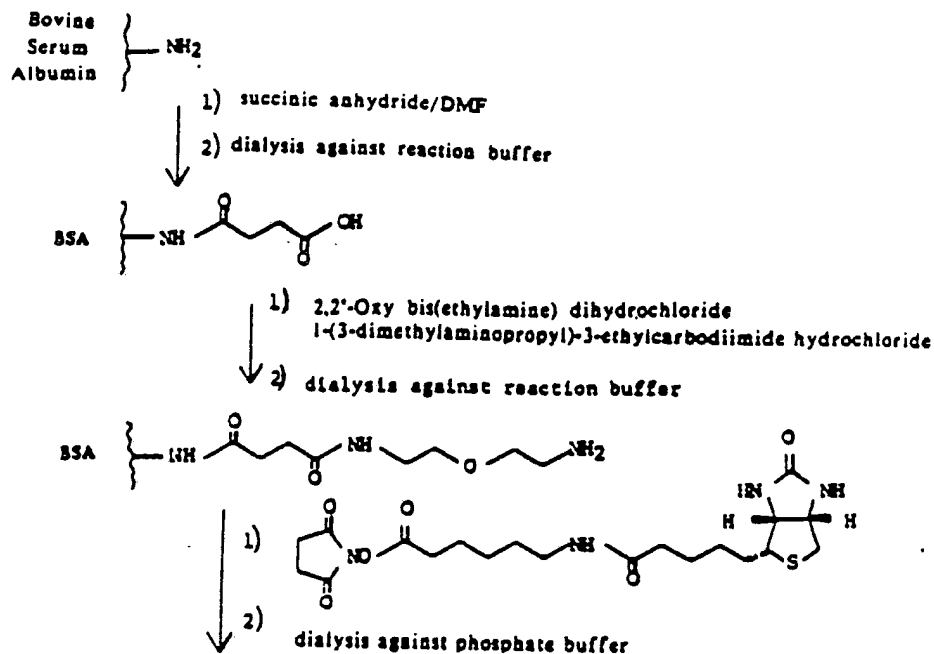
FIG. 6 is a semilog plot illustrating amounts of polymerase chain reaction (PCR) product determined by the dual probe assay.

15        FIG. 7 is a graph of the efficiency of the PCR determined using the dual probe assay.

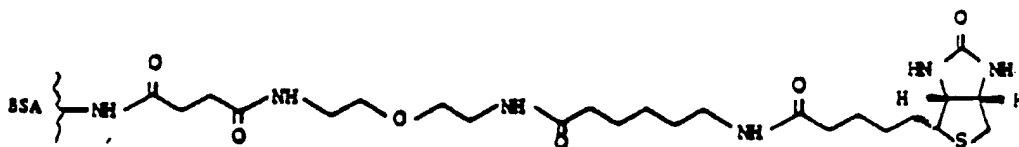
FIG. 8 is standard curves for the determination of MuIgG by sequential and simultaneous procedures.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

A preferred capture membrane is made according to the following reaction scheme.



A compound of the formula:



5. wherein 5-20 biotins with the extended linking group are bound to BSA.

-14-

Example 1Preparation Of Capture Membrane

A pH 8.2 reaction buffer was prepared by dissolving 8.40g sodium bicarbonate and 8.76g sodium chloride in sufficient distilled water to make 1L of solution.

10.00g Bovine Serum Albumin (BSA) was dissolved in 250mL of pH 8.2 reaction buffer and the resulting BSA solution dialyzed exhaustively against reaction buffer with a dialysis filter. The volume after dialysis was still 250mL.

The BSA concentration, as determined by absorbance at 280nm at this point was 40mg/mL. A TNBS assay performed on this BSA solution indicated 18 free amino groups per BSA. TNBS is a 2,4,6-trinitrobenzene sulfonic acid acid/sulfite based assay for free amino groups in a protein which is a modification of the procedure described by Palmer et al., Clin. Chem., 15: 891-901, 1969. An aliquot was removed for later TNBS assays. To the BSA solution was then added 6.32g of  $K_2CO_3$  and the resulting solution was stirred vigorously as a solution of 3.34g succinate anhydride in 83.5mL DMF was added slowly over 14 minutes. The resulting solution was then stirred for an additional 5 minutes and allowed to stand at room temperature for one hour. The solution was then exhaustively dialyzed against reaction buffer and concentrated to 250mL using a Minitan concentrator.

The BSA concentration at this point was determined by absorbance to be 40mg/mL. A TNBS assay performed on the succinylated-BSA solution indicated less than one free amino group per succinylated-BSA.

At this point, 25mL of a 1M solution of 2,2'-Oxy bis(ethylamine) dihydrochloride (pH 8.2) in reaction buffer was added to the stirred BSA solution. Next, 6.39 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was added to the vigorously stirred BSA solution. Stirring was stopped after all of the carbodiimide had dissolved and the resulting solution covered and incubated at room temperature for one hour.

**SUBSTITUTE SHEET**



-15-

The solution was then exhaustively dialyzed against reaction buffer and concentrated to 250mL using a Minitan® (Millipore) concentrator.

The BSA concentration was again determined by absorbance to be  
5 40mg/mL. A TNBS assay run on the above prepared BSA-Oxy bis(ethylamine) solution indicated the presence of 15 free amino groups. A TNBS assay performed on the final dialysis buffer indicated sufficient dialysis of free amines from the BSA solution.

To the BSA solution was then added 1.51g succinimidyl 6-  
10 (biotinamido) hexanoate and the solution was stirred vigorously for 5 minutes after which it was covered and incubated for one hour at room temperature.

The biotinylated-BSA solution was exhaustively dialyzed against a pH 7 phosphate buffer. The phosphate buffer was prepared as  
15 follows: 4.97g of dibasic sodium phosphate, 2.07g of monobasic sodium phosphate and 6.77g of sodium chloride were dissolved in sufficient distilled water to make 1L of solution.

The concentration of biotinylated-BSA in solution after dialysis was determined by absorbance to be 15mg/mL.

20 A TNBS assay performed on the biotinylated-BSA solution indicated the presence of three free amino groups. The difference between the three free amino groups on the biotinylated-BSA and 15 on the BSA - Oxy bis(ethylamine) is 12. This is the number of biotin molecules in the biotinylated-BSA.

25 0.8 $\mu$  nitrocellulose membrane or Immobilon membrane was cut into sheets measuring 20cm by 30cm. 100mL biotin immobilizing solution having 5mg biotinylated BSA/mL phosphate buffer was placed in a trough.

The membrane is immersed into the protein solution. The wetted  
30 membrane was placed on the bottom of a glass dish, the dish inverted and the membrane incubated for 45 minutes. 500mL of phosphate buffer was added to the dish and incubated for 15 minutes. This solution was then decanted.

35 A 0.1% glutaraldehyde fixing solution was prepared by adding sufficient phosphate buffer to 4mL of a 25% glutaraldehyde solution to make 1L. 500mL of this glutaraldehyde fixing solution was then

-16-

added to the dish containing the nitrocellulose membrane, and the contents were incubated at room temperature for two hours. A solution of 0.1 M ethanolamine pH 9.5 was added to a dish containing the Immobolin membrane and the contents were incubated at room temperature overnight.

After decanting the fixing solution or the ethanolamine, 500mL phosphate buffer was added and the contents incubated for 15 minutes. The solution was again decanted, 500mL distilled water added and the contents incubated for 15 minutes.

The water incubation was repeated once. The membrane was then inserted between two sheets of Whatman 3MM blotting paper to remove excess liquid and upon removal from the blotting paper was placed between fiberglass screen material. The screen holding the membrane was placed into a convection oven at 65°C for 15 minutes.

The membrane was removed from the oven and placed into a vacuum desiccator at room temperature for storage.

#### Analysis of the Capture Efficiency of Membrane Coated with Biotin

The characteristics of binding at various flow rates of streptavidin to a biotin coated membrane (BXL-BSA) and of biotinylated BSA to a streptavidin coated membrane is shown in Figure 1. Nitrocellulose membrane was coated with either streptavidin or BXL-BSA so as to allow the binding of approximately equivalent amounts of its corresponding binding partner. Three hundred uL of radiolabeled streptavidin or BXL BSA was filtered through the appropriate membrane at various flow rates. The radioactivity captured on each membrane at the slowest flow rate was assigned 100% capture. The percentage of counts captured is plotted as a percentage of maximum capture against the log of the flow rate.

It is possible that the maximum flow rate tested was still too fast for the streptavidin membrane to capture 100% of the BXL-BSA since the capture curve had not begun to level off. However the biotin coated membrane was able to capture 95% of the streptavidin with a flow rate of 75µL/minute whereas for the streptavidin membrane

-17-

a flow rate of 17 $\mu$ L/minute was needed to capture the same percentage of BXL-BSA.

The effect of biotin density (determined by the biotin/BSA ratio referred to as hapten number) on the capture of streptavidin is shown in Figure 2. Two types of membranes were tested at various flow rates: 0.45 nitrocellulose [NC] and 0.65 $\mu$  Immobilon [IM] membrane. The latter, manufactured by Millipore Laboratories is a polyvinylidene difluoride based membrane which is coated with a hydrophilic polymer and is activated to allow covalent protein attachment. The membranes were coated with bovine serum albumin (BSA) which was labeled to various extent with biotin. The NC and IM membranes contained approximately 10  $\mu$ g/cm<sup>2</sup> and 2 $\mu$ g/cm<sup>2</sup> of BSA respectively. The numbers in parenthesis refer to the time taken for 300 $\mu$ L of <sup>125</sup>I-streptavidin to flow through the membrane. The figure shows that the higher the biotin density on the BSA the better the capture of streptavidin on the membrane. This is more evident on the IM membrane. The greater effect on the IM membrane is due to the lower amount of protein on the membrane. This can be concluded from the next figure (Fig. 3).

The effect of the protein loading on the ability of nitrocellulose membrane coated with biotin-BSA to capture streptavidin is shown in Figure 3. Biotin-BSA, with a hapten number of 16.3, was immobilized onto nitrocellulose membrane at various concentrations. The X axis indicates the concentration of biotin-BSA employed to coat the membrane. The amount of biotin-BSA on the membrane is approximately 2.5, 5, 10, 30, 50  $\mu$ g/cm<sup>2</sup>. Three hundred  $\mu$ L or <sup>125</sup>I-streptavidin was filtered through the membrane in 1.5 minutes and 5 minutes. The Y-axis represents the counts captured by the membrane as a percentage of the maximum counts bounds. As can be seen in the figure, the amount of biotin-BSA on the membrane also plays a role in the capture efficiency of streptavidin on the membranes.

The preceding figures demonstrate several key aspects of the invention: (1) Given equal binding capacities and at a given flow rate, the biotin coated membrane is more efficient at capturing streptavidin than the streptavidin coated membrane is at capturing

-18-

biotin labeled protein. (2) Capture efficiency of the biotin membrane is determined by the biotin density on the membrane. The biotin density is controlled by both the hapten number on the BSA and by the amount of biotin-BSA on the membrane.

5                    Use of Biotin Membrane in Assay for TSH

A pH 7.0 wash buffer was prepared to contain 100mM sodium phosphate, 150mM NaCl, 0.1% bovine gamma globulin and 0.05% of a surfactant such as polyoxyethylene sorbitan monooleate.

10            A nitrocellulose membrane was coated with biotinylated BSA (BXL-BSA) as described above. One square centimeter of the biotinylated membrane was mounted with transfer tape over a 6mm diameter hole of a 1cm x 10cm vinyl acetate stick.

15            The stick containing the membrane was placed into a filter unit, the upper portion of which is a funnel shaped cartridge having a 4mm diameter opening that forces liquid to flow through the membrane.

20            A pH 7.4 conjugate-sample dilution buffer was prepared to contain 10mM sodium phosphate, 150mM NaCl, 10mM Na<sub>2</sub>SO<sub>3</sub>, 1mM 2-mercaptoethanol, 0.01% NaN<sub>3</sub>, 0.1% bovine gamma globulin, 0.05% of a surfactant such as polyoxyethylene mono-oleate, 0.25% octoxynol, 1mM EDTA. 2μg/mL streptavidin - anti-thyroid stimulating hormone and 2μg/mL urease - anti-thyroid stimulating hormone were added to the buffer solution immediately prior to running the assay.

25            750μL conjugate-sample dilution buffer was added to 250μL of serum and the resulting mixture incubated in a covered plastic tube for 1 hour at 37°C.

450μL of this sample-conjugate mixture was filtered under vacuum via syringe pump through the mounted biotinylated membrane in three minutes.

30            The membrane was washed by filtering 500μL of the pH 7.0 wash buffer through it in approximately 1 minute.

35            The stick was then removed from the filter unit and inserted into a sensor assembly having a silicon wafer. See the device of U.S. Patent 4,591,550. The filtration area of the stick matched the light interrogated portion of the silicon wafer. Only one site of the reader was used and the plunger position was set at 70μ from the

-19-

silicon wafer surface. The reaction was monitored for 150 seconds for low signals (250 $\mu$ V/sec) and for 50 seconds for higher signals. Figure 4 illustrates the typical results for TSH determination.

#### Example 2

##### 5                    The Effect of Biotin Attachment Arm On The Recovery of Spiked TSH From Pooled Sera

	<u>Capture Ligand</u> <u>Recovery</u>	<u>Sample</u>	<u>Rate %</u>
	Biotin-BSA	Buffer	1733 "100"
10	Biotin-BSA	Sera	191 11
	BXL-BSA	Buffer	2592 "100"
	BXL-BSA	Sera	2731 105

The table above indicates improvement in recovery of TSH from serum when the long chain form of biotin-BSA (BXL-BSA) is used compared with short-chain biotin-BSA (Biotin-BSA). The above results were obtained with the standard assay protocol described above. Either 1mL pooled sera (sera) of conjugate-sample dilution buffer (buffer) was spiked with 1ng of TSH. Samples were normalized to the rates of the buffer samples (% recovery) for each type of membrane. In addition to the greater recovery in serum, the higher signal in buffer indicates a greater overall capture efficiency of the conjugate complex with BXL-BSA than with short chain biotin.

#### Example 3

##### DNA Assay Dose-Response

25 Membrane: biotin-BSA coated nitrocellulose membrane (0.8 $\mu$  pore size).  
DNA sample: 0, 5, 10, 25, 50 pg of single-stranded Calf thymus DNA in 200 $\mu$ l of phosphate buffered saline (PBS).  
Reagent: 1 $\mu$ g/ml Streptavidin, 1ng/ml SSB-urease, 10ng/ml biotin-anti-DNA, 1% BSA in 10mM Tris-HCl buffer, 1mM EDTA (pH 7.4).  
30 Assay Protocol: 200 $\mu$ l of DNA sample was incubated with 500 $\mu$ l of reagent at 37° for 30 minutes. The mixture was filtered through the biotin-BSA coated membrane at a flow rate of about 100 $\mu$ l/min. The membrane was then washed with 1cc of pH 5 wash buffer (1mM sodium acetate, 0.1M NaCl, 0.05% polyoxyethylene mono-oleate) at a maximum  
35 flow rate of about 6ml/min). After washing, the membrane was

-20-

transferred to pH sensor chambers (U.S. Patent 4,591,550), containing the substrate (pH 5 wash plus 100mM urea) and the pH response was read.

### Results

5	<u>DNA (Pg/sample)</u>	<u>Rate of Signal</u>
	<u>(<math>\mu</math>V/sec)</u>	
	0	62 $\pm$ 3
	5	106 $\pm$ 7
	10	156 $\pm$ 10
10	25	310 $\pm$ 1
	50	778 $\pm$ 70

### Example 4

#### Detection Of DNA In Insulin And Granulocyte Macrophage Colony Stimulating Factor (GMCSF) Samples

15 Membrane: biotin-bSA coated nitrocellulose membrane (0.8 $\mu$  pore size).  
Pre-treatment of Porcine Insulin: 10mg/mL insulin (in 10mM tris buffer, 1mM EDTA, pH 8.5) was digested with 100 $\mu$ g/mL of proteinase K at 55°, overnight. 0, 5, and 50 pg of calf thymus DNA were spiked into 1mg of insulin digest. In another set of experiments, 0, 5, and  
 20 50pg of calf-thymus DNA were spiked into PBS solution containing 0.4mg of GMCSF (total volume was 200 $\mu$ l). All samples were heated at 100°C for 5 minutes to inactivate proteinase K and denature DNA to single-stranded. Insulin samples were cooled on ice and GMCSF samples were cooled down to room temperature.

25 200 $\mu$ l of sample was mixed with 500 $\mu$ l of reagent (200ng/ml streptavidin, 2.5ng/ml biotin-SSB, 9 ng/ml urease-anti-DNA; in 1% BSA, 0.25% octoxynol, 10mM Tris-HCl buffer, 1mM EDTA, pH 7.5).

The samples were incubated at 37° for 30 minutes and filtered through the membrane. 1cc of wash solution 5mM sodium phosphate, pH  
 30 7, 0.1M NaCl 0.05% polyoxyethylene sorbitan mono-oleate) was filtered through the membrane twice. The signal was read with a pH sensor of the type described in U.S. Patent 4,591,550.

-21-

Results

	<u>Sample</u>	<u>Rate (<math>\mu</math>V/sec)</u>
	insulin	39
	insulin + 5 pg DNA	56
5	insulin + 50 pg DNA	254
	GMCSF	21
	GMCSF + 5 pg DNA	40
	GMCSF + 50 pg DNA	192

EXAMPLE 5

10

Dual Probe Assay

Dual probe assay mixtures were prepared to contain 1:2 serial dilutions of Hinfl digested pGEM3 starting at 25 ng ( $7.8 \times 10^9$  target molecules) per assay mixture, 2 ng of 5' biotinylated 20mer (CCAGTTACCTTCGGAAAAAG), and 0.5 ng of 5' fluoresceinated 20mer (TAGCTCTTGATCCGGCAAAC) in 100  $\mu$ l of 3X PBSE (a pH 7.4 solution containing 30 mM sodium phosphate, 450 mM NaCl, and 3 mM EDTA) having 0.25% of a surfactant such as polyoxyethylene sorbitan mono-oleate. This sequence of each 20-mer is also found on the same Hinfl fragment of pGEM3. The sample DNA was then denatured by heating the mixture to 100°C for 5 minutes.

The mixture was subsequently incubated at 50 to 55°C for 30 minutes. To the mixture was then added 1 ml of a solution prepared to contain 1  $\mu$ g/ml streptavidin, 8  $\mu$ g/ml anti-fluorescein-urease conjugate in 3X PBSE, 0.25% polyoxyethylene sorbitan mono-oleate, 1 mg/ml BSA and 80  $\mu$ M N-acetylcysteine. This resulting conjugate solution was incubated for 30 minutes at room temperature.

The incubated conjugate solution was then filtered through a biotin coated nitrocellulose membrane mounted on a plastic stick. Next, 1 ml of threshold wash (10 mM sodium phosphate, 100 mM NaCl, 0.05% polyoxyethylene sorbitan mono-oleate, and 0.05% NaN<sub>3</sub>, pH 6.5) was filtered through the membrane after the conjugate solution and the membrane was dipped into 50 ml of threshold wash. The stick was then inserted into a pH sensor chamber of the type described in U.S. Patent No. 4,591,550 containing substrate solution (100 mM urea in

-22-

threshold wash). The pH response was then read yielding the results displayed in Fig. 5.

#### EXAMPLE 6

##### Application of Dual Probe Assay to Polymerase Chain Reaction (PCR)

5

##### 1. Amplification of 0.1 kbp region in pGEM3 using PCR

A polymerase chain reaction (PCR) mixture was prepared to contain 100 mM Tris-HCl (pH 8.3), 10 mM MgCl<sub>2</sub>, 1 mM each dNTP, 1  $\mu$ M primer 1 (CAAAAAAACCACCGGTACCAG), 1  $\mu$ M primer 2 (AGTATTTGGTATCTGCGCTCTG), 10 pg pGEM3 ( $3.15 \times 10^6$  target molecules) previously digested with HinfI. The region to be amplified contains each of the 20-mer sequences described in Example 5. This PCR mixture was incubated at 100°C for 5 minutes.

Immediately after incubation, 2  $\mu$ l of 5U/ $\mu$ l Taq Polymerase (Perkin Elmer) was added to the PCR mixture which was thoroughly mixed and then incubated at 94°C.

Using an Eppendorf thermal cycler, the incubation temperature was cycled between 94°C and 55°C. The thermal cycler was set so that each cycle lasted about 3 minutes. PCR samples were removed from the mixture in the cycler at cycles 13, 15 and 17 when the temperature reached 56°C. These samples were stored at -20°C for later use in the dual probe assay. Blank samples which did not contain pGEM3 DNA were prepared similarly for use as a negative control.

##### 2. Dual Probe Assay using PCR Sample

10  $\mu$ l of a PCR sample prepared above was combined with 90  $\mu$ l of the dual probe assay mixture prepared in Example 5 and the entire 100  $\mu$ l mixture was denatured at 100°C for 5 minutes.

The mixture was subsequently incubated at 50 to 55°C for 30 minutes. To the mixture was then added 1 ml of a solution prepared to contain 1  $\mu$ g/ml streptavidin, 8  $\mu$ g/ml antiluorescein-urease conjugate in 3X PBSE, 0.25% polyoxyethylene sorbitan mono-oleate, 1 mg/ml BSA and 80  $\mu$ M N-acetylcysteine. This resulting conjugate solution was incubated for 30 minutes at room temperature.

The incubated conjugate solution was then filtered through a biotin coated nitrocellulose membrane mounted on a plastic stick.

**SUBSTITUTE SHEET**



-23-

Next, 1 ml of threshold wash (10 mM sodium phosphate, 100 mM NaCl, 0.05% polyoxyethylene sorbitan mono-oleate, and 0.05%  $\text{NaN}_3$ , pH 6.5) was filtered through the membrane after the conjugate solution. The stick mounted membrane was dipped into 50 ml of threshold wash and then inserted into a pH sensor chamber of the type described in U.S. Patent No. 4,591,550 containing substrate solution (100 mM urea in threshold wash). The pH response was then read yielding the results displayed in Figs. 6 and 7.

The specific signal (the difference between the signals from the samples with and without target DNA) increases more than linearly from cycle 13 to cycle 17 (Fig. 6). Figure 3 shows the amount of specific signal from cycles 13, 15, and 17 on a semilog plot. This graph suggests that the amount of specific signal and therefore PCR product, as determined by comparison with the amount of specific signal generated by known amounts of plasmid DNA (e.g., Fig. 5), increases exponentially from cycle 13 to cycle 17. The increase in specific signal per cycle fits a line that implies that the PCR amplification factor is 1.54 per cycle. Since the theoretical PCR factor is 2.00, the apparent efficiency is 77%.

The kind of measurements taken in this experiment have at least two potential uses. First, the determination of amplification factor is useful in experimental optimization of PCR conditions. For example, there is clearly room for further optimization in the experiment described above. Second, the exponential plot shown in Fig. 7 can be used to determine by extrapolation the amount of starting analyte. For example, the amount of nucleic acid from a pathogen, such as HIV, could be determined. This would be useful in assessing the response to a therapeutic regimen.

#### EXAMPLE 7

##### A. Simultaneous and Sequential Determination of Murine IgG

###### 1. Simultaneous Determination Procedure

A mixture was prepared to contain MuIgG, biotinylated-anti-MuIgG, fluoresceinated-anti-MuIgG, streptavidin, and anti-

-24-

fluorescein-urease. This resulting mixture was incubated for 1 hour at 37°C.

Assay buffer was added to the incubated mixture which was then filtered over a period of 15 minutes through a biotin coated nitrocellulose membrane mounted on a plastic stick. The membrane was washed with pH 7.0 phosphate wash buffer over 5 minutes. The stick as then inserted into a pH sensor chamber of the type described in U.S. Patent No. 4,591,550 containing substrate solution (urea in threshold wash). The pH response was then read yielding the results shown in the following table and figure 8.

#### 2. Sequential Determination Procedure

A mixture of MuIgG, biotinylated-anti-MuIgG and fluoresceinated-anti-MuIgG was prepared and incubated for 1 hour at 37°C. To this incubated mixture was then added streptavidin. The resulting mixture containing streptavidin was filtered over a period of 10 minutes through a biotin coated nitrocellulose membrane mounted on a plastic stick. The membrane was then washed with pH 7.0 phosphate wash buffer over 5 minutes.

A solution of anti-fluorescein-urease was next filtered over 5 minutes through the membrane which was subsequently washed over 5 minutes with pH 7.0 phosphate wash buffer. The stick was then inserted into a pH sensor chamber of the type described in U.S. Patent 4,591,550 containing substrate solution (urea in threshold wash). The pH response was then read yielding the results shown in the following table and figure 8.

#### Determination of Murine IgG by Simultaneous and Sequential Procedures

##### Sequential

pg MuIgG	$\mu$ volt/sec	S.D.(n=4)
0	54	3.0
100	124	3.0
500	403	16
1000	758	93

SUBSTITUTE SHEET

-25-

Simultaneouspg MuIgG     $\mu$ volt/sec    S.D. (n=4)

5	0	120	11
	100	209	11
	500	658	24
	1000	1247	112

10

EXAMPLE 8Simultaneous and Sequential Determinations of  
Anti-Human Chorionic Gonadotropin

Human chorionic gonadotropin (HCG) was labeled with biotin to yield biotinylated HCG. Another sample of HCG was labeled with fluorescein to yield fluoresceinated HCG.

## 1. Sequential Determination of Anti-HCG

400 $\mu$ l samples were prepared to contain 0, 4, 20 and 100 ng of anti-HCG. Each sample was combined with 100 $\mu$ l of solution containing 10 ng of biotinylated HCG and 10 ng of fluoresceinated HCG and the resulting 500 $\mu$ l mixtures were incubated for 1 hour at room temperature.

To each incubated mixture was then added 500 $\mu$ l of a 4 ng/ml streptavidin solution and after mixing, were filtered through a biotin coated nitrocellulose membrane mounted on a plastic stick. Each membrane was then washed with 2 ml of pH 7.0 phosphate wash buffer. 500 $\mu$ l of a 4  $\mu$ g/ml solution of urease-anti-fluorescein was filtered through the membrane which was again washed with 2 ml of the phosphate wash buffer.

The sticks were then inserted into a pH sensor chamber of the type described in U.S. Patent No. 4,591,550 containing urease substrate solution. The pH response was read generating the results displayed in the table below.

## 2. Simultaneous Determination of Anti-HCG

100 $\mu$ l samples were prepared to contain 0, 4, 20, and 100 $\mu$ l of anti-HCG. Each sample was mixed with 100 $\mu$ l of the biotinylated-HCG, fluoresceinated HCG solution used in part 1 of this example and with

-26-

100 $\mu$ l of the 4 $\mu$ g/ml urease-anti-fluorescein solution. The resulting mixture was incubated for 1 hour and 20 minutes at room temperature. To each table was added 1 ml of assay buffer and the resulting mixture filtered through a biotin coated nitrocellulose membrane mounted on a plastic stick. The membrane was washed with 2 ml of wash buffer and read in a pH sensor as described above. The results are displayed below.

This sequential antibody assay may be used to measure an immune antibody response to a vaccine. The specific antibody may be detected at concentrations as low as 1%.

#### Sequential and Simultaneous Determination of anti-HCG

		<u>Rate (<math>\mu</math>v/sec)</u>	
<u>Anti-HCG (ng)</u>		<u>Sequential</u>	<u>Simultaneous</u>
15	0	115 $\pm$ 4	120 $\pm$ 15
	4	130 $\pm$ 6	121 $\pm$ 2
	20	187 $\pm$ 7	165 $\pm$ 7
	100	500 $\pm$ 20	271 $\pm$ 17

#### EXAMPLE 9

##### DNA Dose Response Assay-Alternate Configuration

20 DNA samples were prepared to contain 0, 5, 10, 25, 50, 100, 150 and 200 pg of single-stranded DNA in 500 $\mu$ L of phosphate buffered saline. A dose-response reagent was prepared to contain 5pg/mL streptavidin, 2.25 ng/mL biotinylated single-stranded binding protein, and 93.8 ng/mL anti-DNA urease in Tris-EDTA formulation buffer. The 500  $\mu$ L DNA samples were heat-denatured at 95°C for 10 minutes and subsequently cooled and combined with 1.0mL of dose-response reagent. These mixtures were incubated at 37°C for 1 hour and each was filtered through a biotin-BSA coated nitro-cellulose membrane mounted on a plastic stick. Each membrane was washed with pH 6.5, 10mm phosphate buffer and the stick inserted into a pH sensor chamber of the type described in U.S. Patent No. 4,591,550 containing urease-substrate. The pH response was read generating the data in the table below.

**SUBSTITUTE SHEET**

-27-

Results

	pg DNA	Rate ( $\mu$ volt\sec, mean of 8 tests)	Standard Deviation	Coefficient of Variance (%)
5	0	76.3	12.7	16.0
	5	148	15.6	13.5
	10	222	35.2	15.9
10	25	488	23.3	4.6
	50	981	50.9	5.2
	100	2048	61.3	3.0
	150	3145	112.6	3.5
	200	4662	189.5	4.1

15        From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

-28-

## WHAT IS CLAIMED IS:

1. A capture membrane comprising a porous filter membrane having a hapten bound directly or indirectly to the membrane wherein specifically binding complexes in solution having an anti-hapten  
5 bound to a binding member of the specifically binding complex are removed from a solution by the hapten on the capture membrane as the solution flows through the membrane.
2. A capture membrane according to Claim 1 wherein the hapten is modified with an extended linking group bound directly or  
10 indirectly to the membrane.
3. A capture membrane comprising a porous filter membrane having biotin bound directly or indirectly to the membrane wherein specifically binding complexes in solution having avidin or streptavidin bound to a binding member of the specifically binding  
15 complexes are removed from the solution by the biotin on the capture membrane as the solution flows through the membrane.
4. A capture membrane according to Claim 3 wherein the biotin with an extended linking group is bound to a protein which is adhered to the porous filter membrane and thereby indirectly binds biotin to  
20 the porous filter membrane.
5. A capture membrane according to Claim 4 wherein the protein is bovine serum albumin.
6. A capture membrane according to Claim 5, wherein about 5 to  
20 biotin molecules are bound to each bovine serum albumin molecule.
- 25 7. An assay which comprises the steps of:
  - (a) forming in solution a specifically binding complex between binding members and the substance to be determined wherein one binding member has bound to it an anti-hapten and another binding member having a detectable label;
  - 30 (b) filtering the solution containing the specifically binding complex through a porous filter membrane hapten bound directly or indirectly to the membrane wherein binding between the hapten and anti-hapten captures the specific binding complex on the membrane; and
  - 35 (c) detecting the label attached on the membrane.

-29-

8. An assay according to Claim 7 wherein the hapten is modified with an extended linking group bound directly or indirectly to the membrane.

9. An assay which comprises the steps of:

5 (a) forming in solution a specifically binding complex between binding members and the substance to be determined wherein one binding member has bound to it avidin or streptavidin and another binding member having a detectable label;

10 (b) filtering the solution containing the specifically binding complex through a porous filter membrane having biotin bound directly or indirectly to the membrane wherein binding between the biotin and avidin or streptavidin captures the specific binding complex on the membrane; and

15 (c) detecting the label attached on the membrane.

10. An assay according to Claim 9 wherein the biotin is modified with an extended linking group bound directly or indirectly to the membrane.

20 11. An assay according to Claim 10 wherein the biotin with an extended linking group is bound to a protein which is adhered to the membrane and thereby indirectly binds biotin to the porous filter membrane.

12. An assay according to Claim 11, wherein the binding members are antibodies which bind to the substance to be determined.

25 13. An assay according to Claim 12, wherein the label is an enzyme.

14. An assay for total DNA comprising the steps of:

(a) denaturing the DNA to be detected to single-stranded DNA;

30 (b) forming in solution a specifically binding complex between the single-stranded DNA to be detected, labeled anti-DNA or labeled SSB, which specifically binds to the single-stranded DNA to be detected, and biotinylated anti-DNA or SSB which specifically binds to the DNA to be detected and which is  
35 further bound to avidin or streptavidin;

-30-

(c) filtering the solution through a porous membrane having biotin bound directly or indirectly to the membrane; and

(d) detecting the labeled anti-DNA or labeled SSB attached on the membrane.

5        15. An assay according to Claim 14, wherein the biotin is modified with an extended linking group bound directly or indirectly to the membrane.

10        16. An assay according to Claim 14, wherein the porous membrane is nitrocellulose, cellulose acetate, or polyvinylidene difluoride polymer coated with a hydrophilic polymer.

17. An assay according to Claim 16 wherein the label is an enzyme.

18. An assay according to Claim 17, wherein the enzyme is urease or horseradish peroxidase or alkaline phosphatase.

15        19. An assay for an antigen comprising the step of:

(a) forming in solution a specifically binding complex between the antigen to be detected, enzyme labeled antibody to the antigen; and an antibody to the antigen where avidin or streptavidin is bound to the antibody;

20        (b) filtering the solution containing the specifically binding complex through a porous filter membrane having a protein with a plurality of biotins bound to the protein dispersed on the porous filter membrane whereby the biotin binds the specifically binding complex to the membrane; and

25        (c) detecting the enzyme on the membrane.

20. An assay for an antigen according to Claim 19, wherein the biotins are modified with extended linking groups bound directly or indirectly to the membrane.

30        21. In an assay where a specifically binding complex is removed from solution by a membrane which binds the specifically binding complex formed by specific binding, the improvement comprising a porous filter membrane having a hapten bound directly or indirectly to the porous filter membrane and an anti-hapten bound to a binding member of the specifically binding complex.



-31-

22. An assay according to Claim 21, wherein the hapten is modified with an extended linking group bound directly or indirectly to the porous filter membrane.

23. An assay according to Claim 21, wherein the hapten is biotin and the anti-hapten is streptavidin.

24. An assay for rare RNA or rare DNA comprising the steps of:

(a) denaturing the rare RNA or DNA to be detected to single-stranded RNA or DNA;

(b) forming in solution a specifically binding complex between the single-stranded RNA or DNA to be detected, labeled oligonucleotide probe, which binds in a sequence-specific fashion to the single-stranded RNA or DNA to be detected, and biotinylated oligonucleotide probe which specifically binds to the RNA or DNA to be detected and which is further bound to avidin or streptavidin;

(c) filtering the solution through a porous membrane having biotin bound directly or indirectly to the membrane; and

(d) detecting the labeled oligonucleotide probe attached on the membrane.

25. An assay according to Claim 24, wherein an extended linking group binds the biotin directly or indirectly to the membrane.

26. An assay according to Claim 24, wherein the porous membrane is nitrocellulose, cellulose acetate, or polyvinylidene difluoride polymer coated with a hydrophilic polymer.

27. An assay according to Claim 26 wherein the label is an enzyme.

28. An assay according to Claim 27, wherein the enzyme is urease or horseradish peroxidase or alkaline phosphatase.

29. An assay according to Claim 24, wherein the biotinylated oligonucleotide probe comprises biotin modified with an extended linking group bound to the oligonucleotide.

30. In a quantitative polymerase chain reaction assay, the improvement comprising the steps of:

(a) removing a plurality of samples of amplified DNA from a polymerase chain reaction at various times;

-32-

- 5 (b) forming in solution a specifically binding complex between the amplified DNA to be detected, labeled oligonucleotide probe, which specifically binds to the amplified DNA to be detected, and biotinylated oligonucleotide probe which specifically binds to the amplified DNA to be detected and which is further bound to avidin or streptavidin;
- (c) filtering the solution through a porous membrane having biotin bound directly or indirectly to the membrane;
- 10 (d) detecting the labeled oligonucleotide probe attached on the membrane;
- (e) determining the amount of DNA formed by the polymerase chain reaction;
- (f) extrapolating the quantity of DNA present prior to amplification.
- 15 31. An assay according to Claim 29, wherein the porous membrane is nitrocellulose, cellulose acetate, or polyvinylidene difluoride polymer coated with a hydrophilic polymer.
32. An assay according to Claim 30 wherein the label is an enzyme.
- 20 33. An assay according to Claim 31, wherein the enzyme is urease or horseradish peroxidase or alkaline phosphatase.
34. An assay for an antigen comprising the steps of:
- (a) forming in solution a specifically binding complex between the antigen to be detected, enzyme labeled antibody to the antigen, an antibody to the antigen where biotin is bound to the antibody, and streptavidin or avidin;
- 25 (b) filtering the solution containing the specifically binding complex through a porous filter membrane having a protein with a plurality of biotins bound to the protein dispersed on the porous filter membrane whereby the biotin binds the specifically binding complex to the membrane; and
- 30 (c) detecting the enzyme on the membrane.
- 35 35. An assay for an antigen according to Claim 34, wherein an extended linking group binds the biotins dispersed on the porous filter membrane directly or indirectly to the protein dispersed on the porous filter membrane.

-33-

36. An assay according to Claim 35, wherein the biotin bound to the antibody is modified with an extended linking group bound to the antibody.

37. An assay according to Claim 36, wherein the porous membrane  
5 is nitrocellulose, cellulose acetate, or polyvinylidene difluoride polymer coated with a hydrophilic polymer.

38. An assay according to Claim 37 wherein the label is an enzyme.

39. An assay according to Claim 38, wherein the enzyme is  
10 urease or horseradish peroxidase or alkaline phosphatase.

40. An assay for an antibody comprising the steps of:

(a) forming in solution a specifically binding complex  
between the antibody to be detected, enzyme labeled antigen to  
the antibody; and an antigen to the antibody where biotin is  
15 bound to the antibody, and avidin or streptavidin.

(b) filtering the solution containing the specifically  
binding complex through a porous filter membrane having a  
protein with a plurality of biotins bound to the protein  
dispersed on the porous filter membrane whereby the biotin binds  
20 the specifically binding complex to the membrane; and

(c) detecting the enzyme on the membrane.

41. An assay for an antibody according to Claim 40, wherein the  
biotins bound to the protein are modified with extended linking  
groups bound directly or indirectly to the protein.

25 42. An assay according to Claim 41, wherein the biotin bound to  
the antigen is modified with an extended linking group bound to the  
antigen.

43. An assay according to Claim 42, wherein the porous membrane  
is nitrocellulose, cellulose acetate, or polyvinylidene difluoride  
30 polymer coated with a hydrophilic polymer.

44. An assay according to Claim 43 wherein the label is an  
enzyme.

45. An assay according to Claim 44, wherein the enzyme is  
urease or horseradish peroxidase or alkaline phosphatase.

35 46. An assay which comprises the steps of:

-34-

(a) forming in solution a specifically binding complex between binding members and the substance to be determined wherein one binding member has bound to it a hapten and another binding member having a detectable label;

5 (b) filtering a solution containing an anti-hapten through a porous filter membrane having hapten bound directly or indirectly to the membrane, wherein binding between the hapten and the anti-hapten captures the anti-hapten on the membrane;

10 (c) filtering the solution containing the specifically binding complex through the porous filter membrane having anti-hapten bound to the membrane wherein binding between the hapten in the specifically binding complex and anti-hapten captures the specific binding complex on the membrane;

(d) detecting the label attached on the membrane.

15 47. An assay according to Claim 46, wherein the label is an enzyme.

48. An assay according to Claim 47 wherein the enzyme is detected by an electrode.

20 49. An assay according to Claim 48 wherein the electrode is a semiconductor.

50. An assay according to Claim 46 wherein the hapten bound to the membrane is modified with an extended linking group bound directly or indirectly to the membrane.

51. An assay which comprises the steps of:

25 (a) forming in solution a specifically binding complex between binding members and the substance to be determined wherein one binding member has bound to it biotin and another binding member having a detectable label;

30 (b) filtering a solution containing avidin or streptavidin through a porous filter membrane having biotin bound directly or indirectly to the membrane, wherein binding between the biotin and avidin or streptavidin captures the avidin or streptavidin on the membrane;

35 (c) filtering the solution containing the specifically binding complex through the porous filter membrane having avidin or streptavidin bound to the membrane wherein binding between

**SUBSTITUTE SHEET**

-35-

the biotin in the specifically binding complex and avidin or streptavidin captures the specific binding complex on the membrane; and

(d) detecting the label attached on the membrane.

5        52. An assay according to Claim 51 wherein the biotin bound to the membrane is modified with an extended linking group bound directly or indirectly to the membrane.

10       53. An assay according to Claim 52 wherein the biotin with an extended linking group is bound to a protein which is adhered to the membrane and thereby indirectly binds biotin to the porous filter membrane.

54. An assay according to Claim 53, wherein the binding members are antibodies which bind to the substance to be determined.

15       55. An assay according to Claim 54, wherein the label is an enzyme.

56. An assay according to Claim 51 wherein the label is detected by an electrode.

57. An assay according to Claim 56 wherein the electrode is a semiconductor.

20       58. A sequential assay which comprises the steps of:

(a) filtering a solution containing an anti-hapten through a porous filter membrane having hapten bound directly or indirectly to the membrane, wherein binding between the hapten and the anti-hapten captures the anti-hapten on the membrane;

25       (b) filtering a solution containing an anti-hapten binding substance modified with binding sites for a substance to be determined through the porous filter membrane whereby the anti-hapten binding substance is captured on the membrane;

30       (c) filtering a solution containing or suspected to contain a substance to be determined through the membrane, whereby the substance to be determined is captured on the membrane;

(d) labeling the substance to be determined with a detectable label; and

35       (e) detecting the label.

**SUBSTITUTE SHEET**

-36-

59. An assay according to Claim 58 wherein the label is an enzyme.

60. An assay according to Claim 59 wherein the enzyme is detected by an electrode.

5 61. An assay according to Claim 60 wherein the electrode is a semiconductor.

62. A sequential assay which comprises the steps of:

10 (a) filtering a solution containing avidin or streptavidin through a porous filter membrane having biotin bound directly or indirectly to the membrane, wherein binding between the biotin and the avidin or streptavidin captures the avidin or streptavidin on the membrane;

15 (b) filtering a solution containing an avidin or streptavidin binding substance modified with binding sites for a substance to be determined through the porous filter membrane whereby the avidin or streptavidin binding substance is captured on the membrane;

20 (c) filtering a solution containing or suspected to contain a substance to be determined through the membrane, whereby the substance to be detected is captured on the membrane;

(d) labeling the substance to be determined with a detectable label; and

(e) detecting the label.

25 63. An assay according to Claim 62 wherein the label is an enzyme.

64. An assay according to Claim 63 wherein the enzyme is detected by an electrode.

30 65. An assay according to Claim 64 wherein the electrode is a semiconductor.

66. An assay according to Claim 62 wherein the biotin bound to the membrane is modified with an extended linking group bound directly or indirectly to the membrane.

**SUBSTITUTE SHEET**

-37-

67. An assay according to Claim 66 wherein the biotin with an extended linking group is bound to a protein which is adhered to the membrane and thereby indirectly binds biotin to the porous filter membrane.

- 5        68. An assay according to Claim 67, wherein the avidin or streptavidin binding members are antibodies which bind to the substance to be determined.

1 / 4

Fig. 1

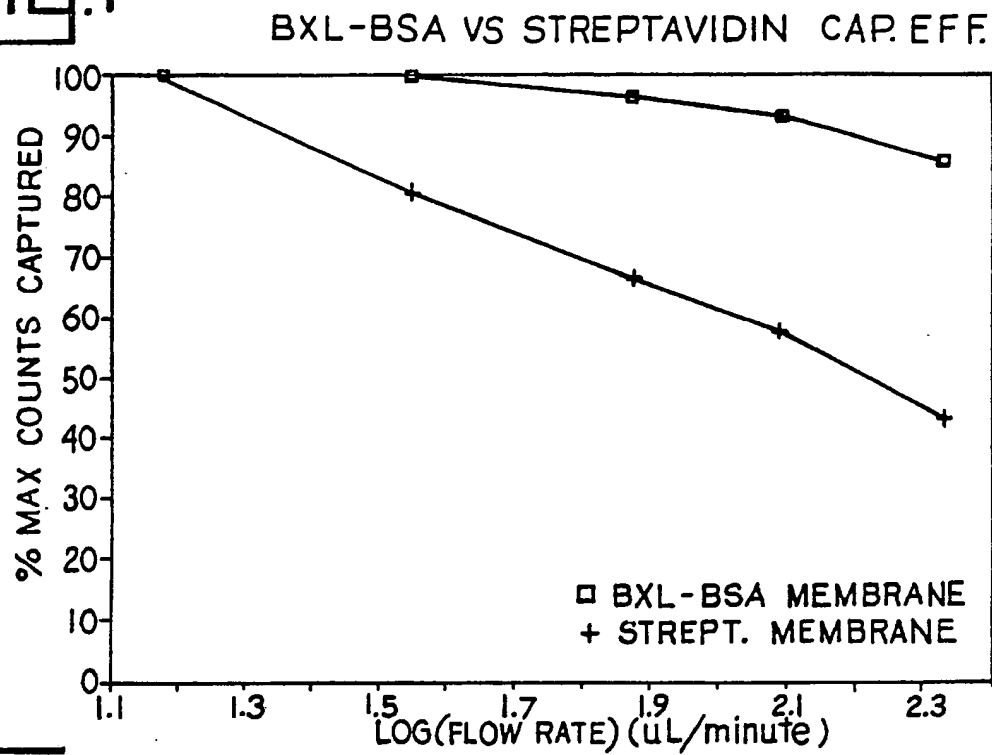
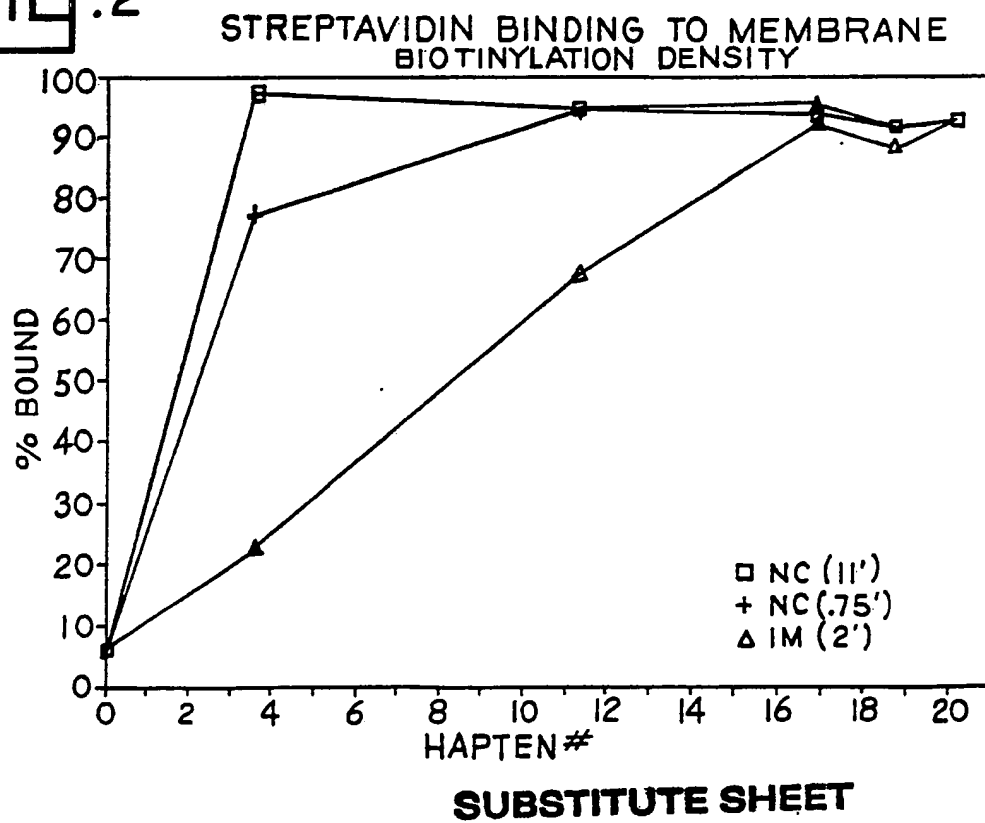


Fig. 2





2 / 4

Fig. 3

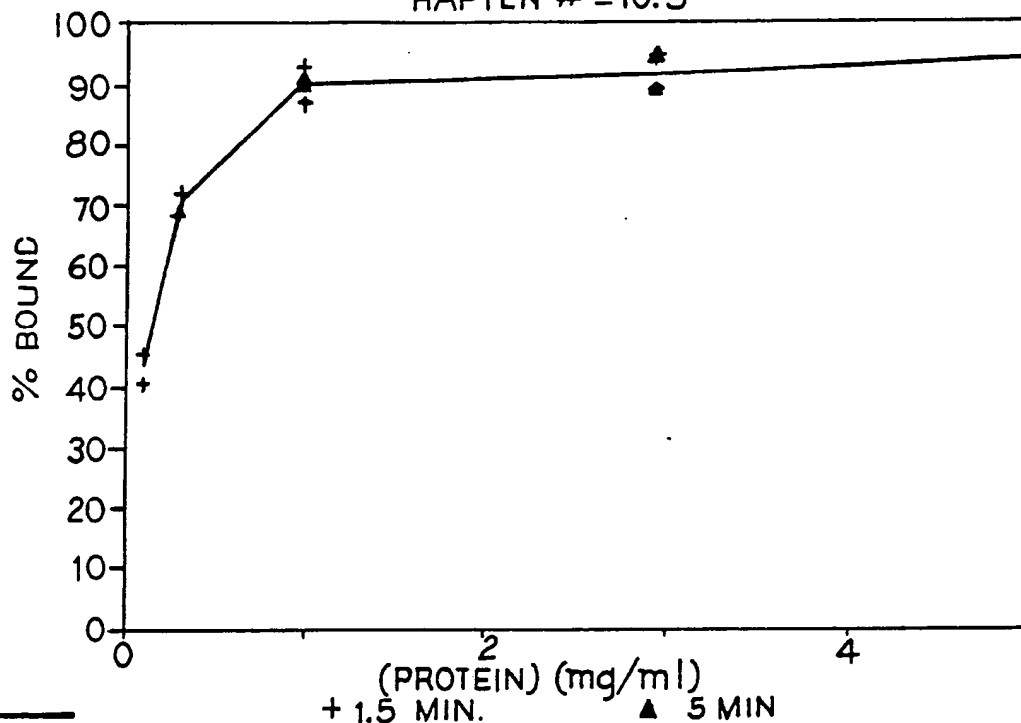
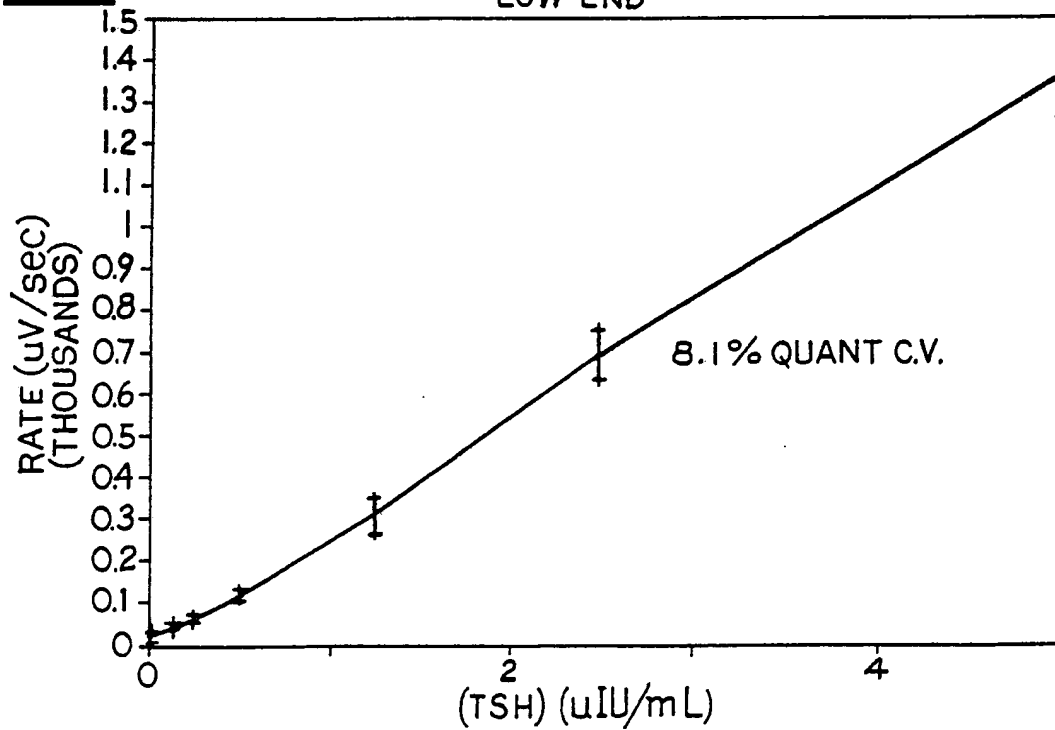
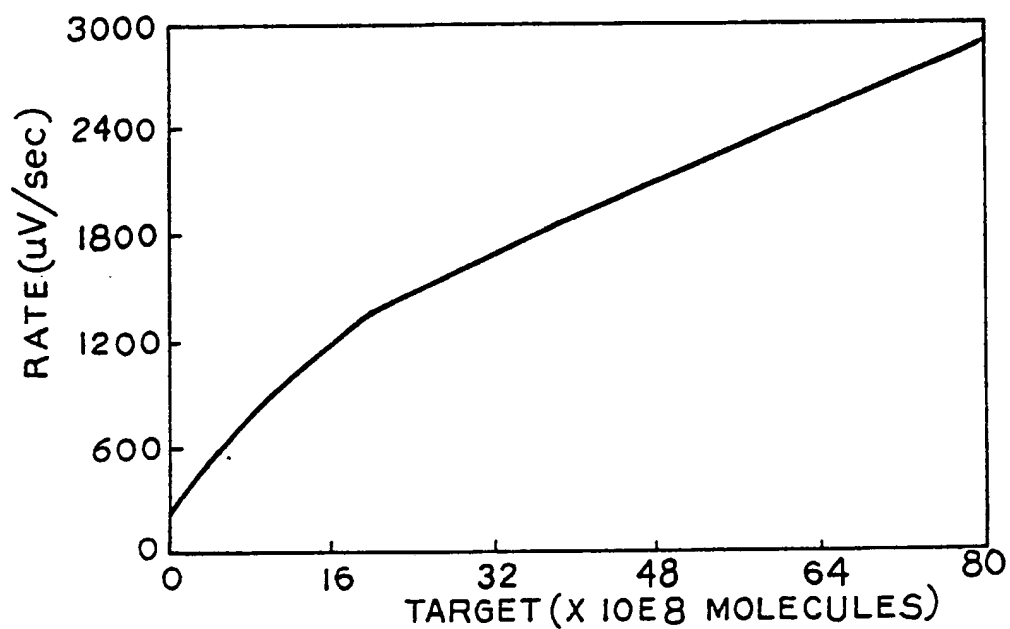
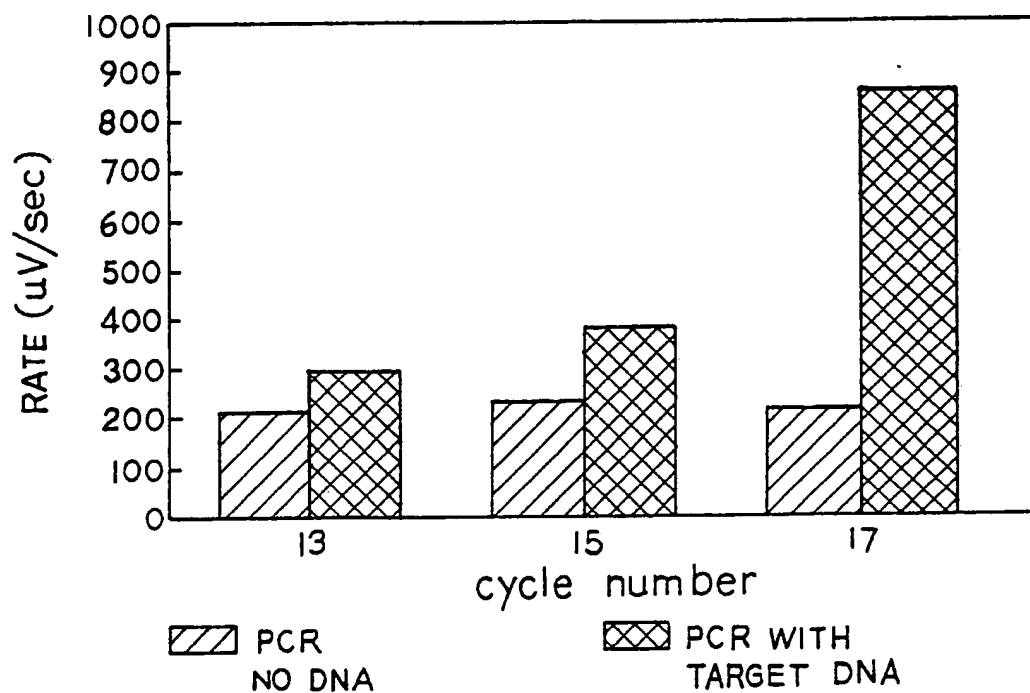
CAPTURE EFFICIENCY OF 0.8  $\mu$  NITROCELLULOSE  
HAPTEN # = 10.3

Fig. 4

TSH SENSOR ASSAY  
LOW END

SUBSTITUTE SHEET

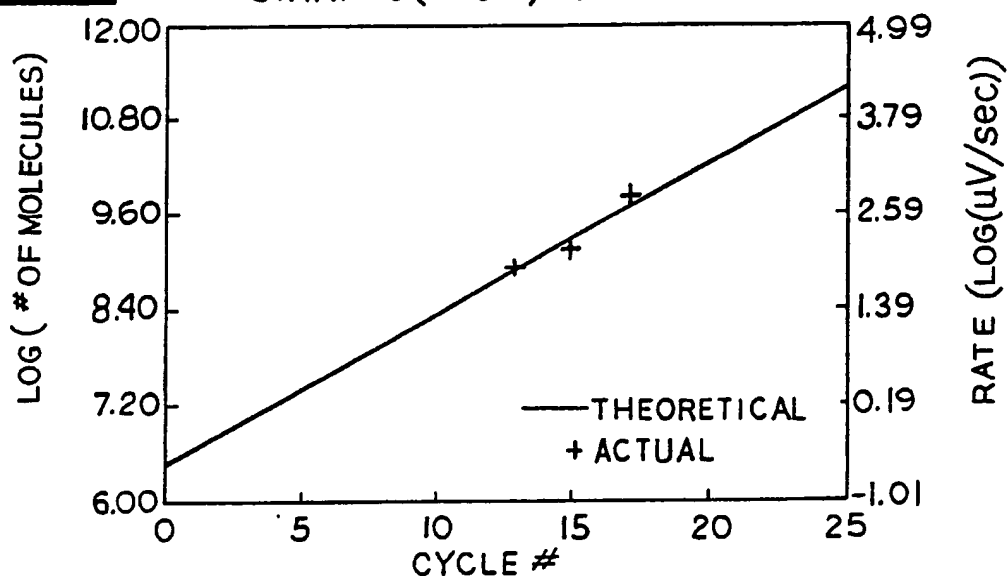
3 / 4

**Fig. 5****DUAL PROBE ASSAY****Fig. 6****DUAL PROBE ASSAY****SUBSTITUTE SHEET**

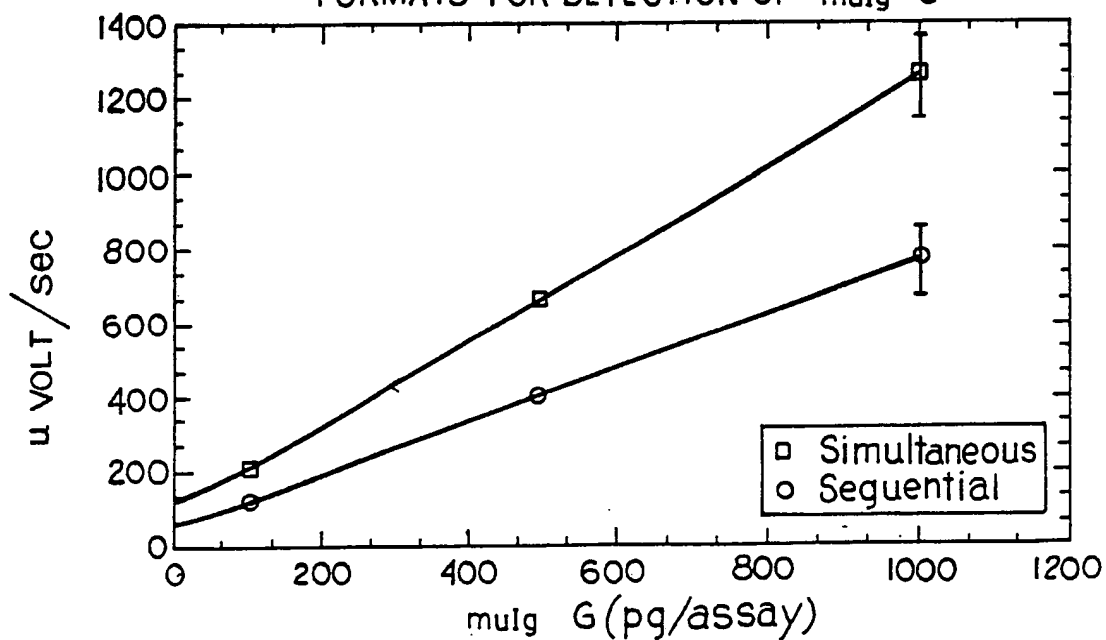
4 / 4

**Fig. 7**

PCR EFFICIENCY OF 77%  
STARTING (TARGET) =  $3.15 \times 10^6$  MOLECULES

**Fig. 8**

COMPARISON OF "SIMULTANEOUS" AND "SEQUENTIAL"  
FORMATS FOR DETECTION OF  $\mu$ lg G

**SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/04320

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: G 01 N 33/53		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
IPC5	G 01 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT*</b>		
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	GB, A, 2199327 (PALL CORPORATION) 6 July 1988, see claims 1,10-23	1-2
Y	--	1-6
X	WO, A1, 85/05451 (HYBRITECH INCORPORATED) 5 December 1985, see claims 1-54, page 11, lines 25-34	1-6
Y	--	7-68
Y	FR, A1, 2601455 (LABORATOIRE DES STALLERGENES) 11 July 1986, see the whole document	3-13,19-68
	--	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 25th January 1990	Date of Mailing of this International Search Report 12. 02. 90	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer C.D. v.d. Vliet	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0201079 (ZAHRADNIK, RICHARD) 12 November 1986, see claims 1-11, page 5, line 7 - page 7 line 6  --	7-68
Y	DE, A1, 3705686 (BOEHRINGER MANNHEIM GMBH) 1 September 1988, see claims 1-7, page 3 line 29-66  --	7-13, 19- 68
A	Patent Abstracts of Japan, Vol 4, No 144, C 27, abstract of JP 55- 92693, publ 1980-07-14 (HEKISUTO JAPAN K.K.)  --  -----	7-13, 19- 68

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

**PCT/US 89/04320**

**SA**

**31864**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on **08/11/89**  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 2199327	06/07/88	EP-A-B- 0173500	05/03/86
		GB-A-B- 2163434	26/02/86
		JP-A- 61124868	12/06/86
		US-A- 4693985	15/09/87
		EP-A- 0280840	07/09/88
		CA-A- 1249781	07/02/89
WO-A1- 85/05451	05/12/85	AU-D- 44017/85	13/12/85
		EP-A- 0180638	14/05/86
		JP-T- 61502214	02/10/86
		US-A- 4632901	30/12/86
		US-A- 4727019	23/02/88
		CA-A- 1257542	18/07/89
FR-A1- 2601455	11/07/86	NONE	
EP-A2- 0201079	12/11/86	AU-D- 57046/86	13/11/86
		JP-A- 62030963	09/02/87
DE-A1- 3705686	01/09/88	EP-A- 0280211	31/08/88
		JP-A- 63229368	26/09/88

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**